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Short communication

Penetration of equine leukocytes by merozoites of *Sarcocystis neurona*

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Abstract

Horses are considered accidental hosts for *Sarcocystis neurona* and they often develop severe neurological disease when infected with this parasite. Schizont stages develop in the central nervous system (CNS) and cause the neurological lesions associated with equine protozoal myeloencephalitis. The present study was done to examine the ability of *S. neurona* merozoites to penetrate and develop in equine peripheral blood leukocytes. These infected host cells might serve as a possible transport mechanism into the CNS. *S. neurona* merozoites penetrated equine leukocytes within 5 min of co-culture. Infected leukocytes were usually monocytes. Infected leukocytes were present up to the final day of examination at 3 days. Up to three merozoites were present in an infected monocyte. No development to schizont stages was observed. All stages observed were in the host cell cytoplasm. We postulate that *S. neurona* merozoites may cross the blood brain barrier hidden inside leukocytes. Once inside the CNS these merozoites can egress and invade additional cells and cause encephalitis.
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1. Introduction

Equine protozoal myeloencephalitis (EPM) caused by *Sarcocystis neurona* is a major neurological syndrome of horses in the Americas (Dubey et al., 2001b). This apicomplexan parasite was known since the early 1970’s but was not named until 1991 when it

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was isolated and grown in cell culture (Dubey et al., 1991). Horses are considered accidental hosts. *S. neurona* schizonts and merozoites are present in the central nervous system (CNS). The Virginia opossum, *Didelphis virginiana*, is the only known definitive host in North America (Dubey and Lindsay, 1998), whereas *Didelphis albiventris* is a host in South America (Dubey et al., 2001a). Nine-banded armadillos (*Dasypus novemcinctus*), domestic cats (*Felis domesticus*), raccoons (*Procyon lotor*), fisher (*Martes pennanti*) and sea otters (*Enhydra lutris*) are natural hosts (Cheadle et al., 2001a; Dubey et al., 2001c,d; Gerhold et al., 2005; Tanhauser et al., 2001). Domestic cats, striped skunks (*Mephitis mephitis*) and raccoons are known experimental intermediate hosts (Butcher et al., 2002; Cheadle et al., 2001b; Dubey et al., 2000, 2001d).

Attempts to reproduce clinical EPM in immunologically normal horses using sporocysts have met with variable success (Cutler et al., 1999, 2001; Fenger et al., 1997; Saville et al., 2001, 2004; Sellon et al., 2004; Sofaly et al., 2002). No *S. neurona* schizonts have been observed in the CNS or other tissues of immunologically normal horses fed sporocysts. Direct inoculation of merozoites in to the cerebral spinal fluid did not produce clinical disease in one study (Lindsay et al., 2000).

Ellison et al. (2004) have recently published an equine model of EPM that produces clinical signs and identifiable parasites in the tissues of experimentally infected horses. Ellison et al. (2004) demonstrated that horse leukocytes co-cultured with *S. neurona* merozoites were infectious for immunocompetent horses. Horses inoculated with these preparations developed EPM. They (Ellison et al., 2004) postulated that merozoites were entering leukocytes and that these infected leukocytes were migrating across the menengies and entering the CNS. The present study was conducted to evaluate the interactions of *S. neurona* merozoites with equine leukocytes using transmission electron microscopy (TEM).

2. Materials and methods

2.1. *Sarcocystis neurona* merozoites

Merozoites of the *S. neurona* SN-37R isolate (Lindsay et al., 2004; Sofaly et al., 2002) were used. African green monkey (*Cercopithecus aethiops*) kidney cells (CV-1 cells, ATTC CCL-70, American Type Culture Collection, Manassas, VA) were grown to confluence in 75-cm^2 plastic cell culture flasks in growth media that consisted of 10% (v/v) fetal bovine serum (FBS) in RPMI 1640 medium supplemented with 100 U penicillin G/ml, and 100 mg streptomycin/ml. Cell cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. Merozoites were collected from infected CV-1 cells and filtered through a sterile 3 μM filter and counted in a hemocytometer prior to inoculation of equine lymphocytes.

2.2. Equine leukocytes

The leukocytes used were collected from a *S. neurona* Western blot negative horse using methods previously described (Witonsky et al., 2003). Briefly, blood was diluted 1:2 with RPMI 1640 media. Diluted blood was layered 2:1 on Lymphoprep 1.077 (Nycomed, Oslo, Norway). All reagents and blood samples were allowed to warm to room temperature (RT) to optimize leukocyte recovery. Blood was centrifuged for 20 min at RT. The buffy coat was collected and washed three times with RPMI 1640 media. Following the second wash, cells were re-suspended and counted by use of an automated cell counter (CASY-1 model TTC cell counter and analyzer system, Sharfe System, GMbH, Reutingen, Germany). Cells were diluted to a concentration of 2×10^6 cells/ml in RPMI 1640 supplemented with 10% fetal calf serum, 50 U/ml penicillin and 50 mg/ml streptomycin. The recoveries of leukocytes following lymphoprep isolation are 85–90% lymphocytes, 3–10% neutrophils and 1–5% monocytes (Witonsky et al., 2003).

2.3. TEM

For TEM, *S. neurona* merozoites (100 μl) were mixed with equine leukocytes from a donor horse (100 μl) at a 2:1 ratio in microfuge tubes, pelleted in a microfuge for 2 min and incubated at 37 °C. The co-cultures were fixed in 3% (v/v) glutaraldehyde in phosphate buffer (PBS, pH 7.4) after incubation at 37 °C for 5 and 30 min. Two additional cultures were re-suspended after 30 min and incubated as a suspension for 1 and 3 days in medium at 37 °C after which they were pelleted in microfuge and fixed for TEM examination. Cell pellets were post-fixed in 1% (w/v) osmium tetroxide in 0.1 M phosphate buffer, dehydrated in a series of ethanols, passed through two changes of propylene oxide, and embedded in Poly/Bed 812 resin (Polysciences Inc., Warrington, PA). Thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss 10CA TEM operating at 60 kV. Digital images were captured using an ATM camera system (Advanced Microscopy Techniques Corp., Danvers, MA). Thick sections were stained with methylene blue-Azure II-Basic fuchsin triple stain (Hayat, 1989) and mounted on glass slides for observation with light microscopy.

3. Results

TEM results are based on examination of 7 micrographs taken at 5 min PI, 6 at 30 min post culture (PC), 10 at 1 day and 18 at 3 days PC. Intracellular merozoites were seen at 5 min PC and at every examination period using TEM (Figs. 1–4). Merozoite appeared to actively penetrate equine leukocytes (Fig. 1). Merozoites were always located inside the host cell cytoplasm and not in a parasitophorous vacuole (Figs. 2 and 3). Multiple penetration of leukocytes was common (Fig. 4) and seen as early as 5 min PC. No schizonts were observed. Degenerate host cells containing merozoites were common 3 days PC. The results from thick sections examined at various times PC were similar: multiple infection was observed but no schizogony occurred.

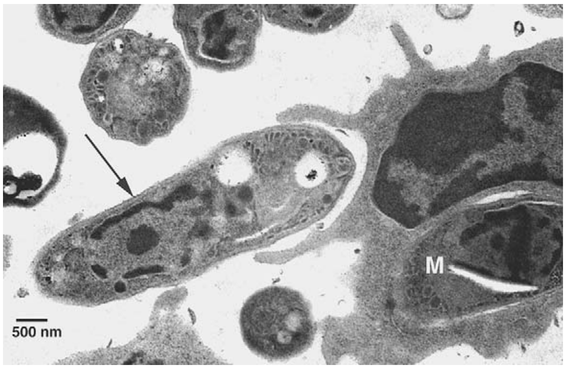

Fig. 1. Transmission electron micrograph an infected equine leukocyte that is in the process of being invaded by a *Sarcocystis neurona* merozoite (arrow) 5 min post culture. Note the intracellular merozoite (M) and absence of a parasitophorous vacuole. Bar = 500 nM.

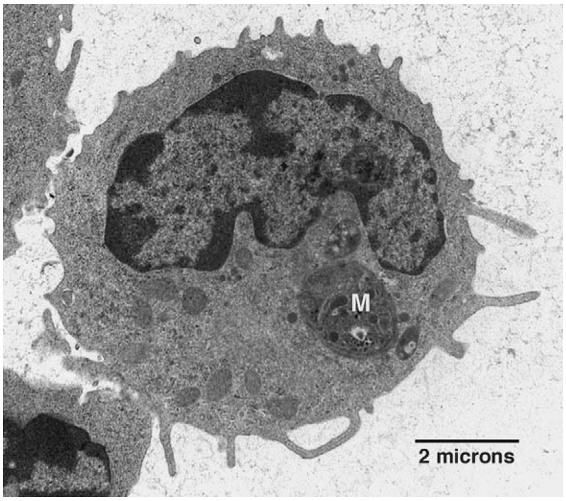

Fig. 2. Transmission electron micrograph of an equine monocyte containing a single *Sarcocystis neurona* merozoite (M) 30 min post culture. Note the absence of a parasitophorous vacuole. Bar = 2 μm.

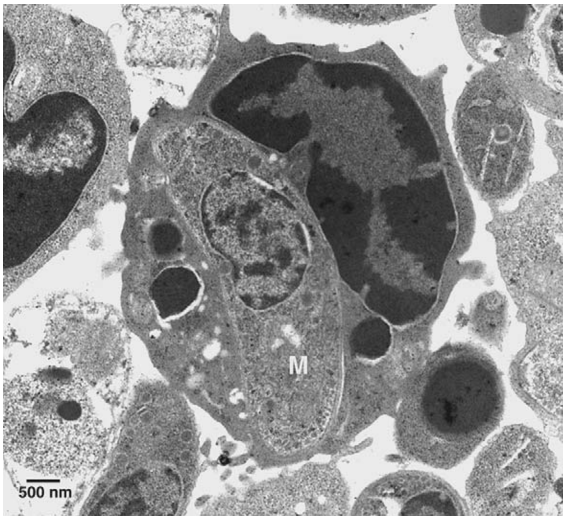

Fig. 3. Transmission electron micrograph of an infected monocyte containing a single *Sarcocystis neurona* merozoite (M) 1 day post culture. Note the absence of a parasitophorous vacuole. Bar = 500 nM.

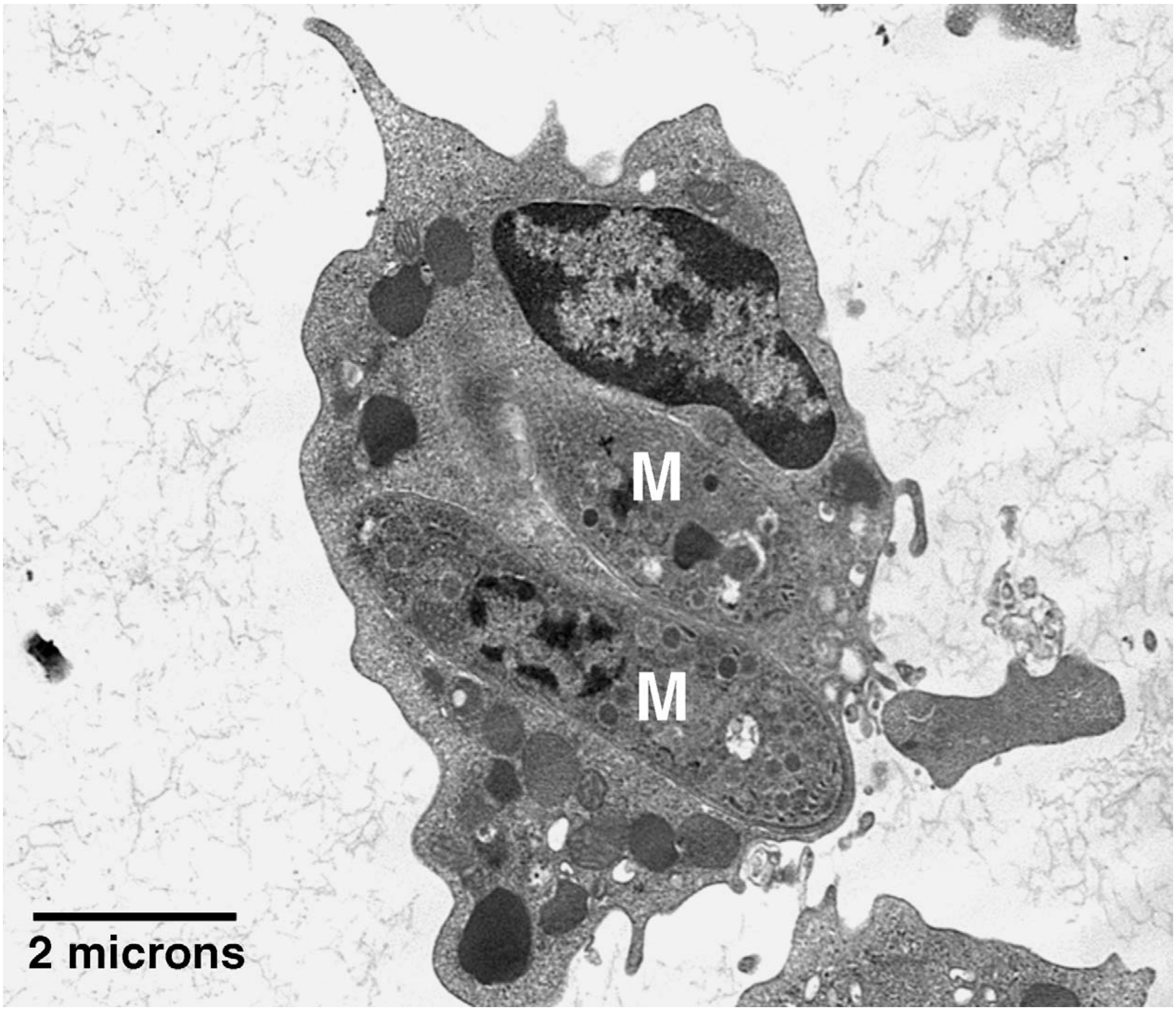

Fig. 4. Transmission electron micrograph of an infected equine monocyte containing two *Sarcocystis neurona* merozoites (M) 1 day post culture. Note the absence of a parasitophorous vacuole. Bar = 2 μm.

4. Discussion

The TEM and light microscopic examinations conducted in this study demonstrate that *S. neurona* merozoites will penetrate and survive in equine lymphocytes for up to 3 days. Invasion of host cells by *S. neurona* merozoites is quick and can occur within 5 min (Fig. 1). The multiple merozoite leukocytes observed in the present study maybe a product of experimental design but multiple infection of host cells in culture occurs commonly with *S. neurona* (Lindsay et al., 1999). Penetration of host cells by coccidian sporozoites, merozoites and bradyzoites is an active process that is distinct from phagocytosis (Dowse and Soldati, 2004; Carruthers and Blackman, 2005) and it is therefore unlikely that phagocytosis was the means of *S. neurona* entrance in to leukocytes in the present study.

It takes 3 days for *S. neurona* merozoites to complete schizogony in mammalian cell cultures (Lindsay et al., 1999). No schizogony occurred during the 3-day observation period and no indication of merozoite development to schizonts occurred indicating that leukocytes will not support schizogony of *S.*

neurona in vitro. *Sarcocystis neurona* will develop in a bovine monocyte cell line in vitro (Ellison et al., 2001) but the conditions for development must not have been present for development in the many infected equine monocytes seen in the present study. Coccidial zoites can enter and leave host cells rapidly (Lindsay et al., 1983; Lindsay and Current, 1984). It is possible that once transported to the central nervous system within leukocytes, that the merozoites can exit the transporting cell and infect additional cells in the central nervous system. Development would then occur in these permissive host cells.

Fayer and Leek (1979) demonstrated that *S. cruzi* could be transmitted by blood transfusion and that merozoites stages could be found in the blood of infected calves. *Sarcocystis neurona* has been isolated from the blood of orally infected immunocompromised foals (Long et al., 2002; Sellon et al., 2004) and immunocompetent horses (Rossano et al., 2005). Sellon et al. (2004) were able to demonstrate *S. neurona* parasitemia in intravenously inoculated immunocompetent horses. These studies clearly indicate that circulation of *Sarcocystis* stages occur in intermediate and accidental hosts. Liang et al. (1998) demonstrated that serum and cerebral spinal fluid from *S. neurona* infected horses could neutralize or inhibit merozoite development in bovine turbinate cell cultures. This suggests that merozoites need to be protected from antibodies while in the equine circulatory system. The present study provides ultrastructural evidence that equine leukocytes can harbor *S. neurona* merozoites and potentially aid in the survival of *S. neurona* merozoites by helping them evade lyses by antibodies. Additional studies on the migratory abilities of *S. neurona* infected leukocytes are needed to determine the importance of these cells in the genesis of equine protozoal myeloencephalitis.

Results of this study indicate that a parasitophorous vacuole is not formed in the host cell even at the earliest stages of penetration. However, it is possible that a parasitophorous vacuole forms and degenerates within 5 min. Speer and Dubey (2001) showed that the schizogony of *S. neurona* was always in direct contact with the host cell cytoplasm without a parasitophorous vacuole membrane. However, Speer and Dubey (2001) examined infected cultures 1 day PC or later.

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